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GENETIC DIFFERENTIATION BETWEEN THE SIBLING AND SYMPATRIC FLOWER-HEAD INFESTING TEPHRITIDS: THE POLYPHAGE, *TRUPANEA NIGRICORNIS* (COQUILLETT), AND THE NARROWLY OLIGOPHAGOUS, *T. BISETOSA* (COQUILLETT) (DIPTERA: TEPHRITIDAE)

KHOUZAMA M. KNIO, RICHARD D. GOEDEN, AND DAVID H. HEADRICK

(KMK) Biology Department, American University of Beirut, P.O. Box 11-0236, Riad El Sohl, Beirut, Lebanon (e-mail: kknio@aub.edu.lb); (RDG) Department of Entomology, University of California, Riverside, CA 92521, U.S.A.; (DHH) Horticulture and Crop Science Department, California Polytechnic State University, San Luis Obispo, CA 93407, U.S.A.

Abstract.—*Trupanea nigricornis* and *T. bisetosa* are sibling, cryptic species that occur in sympatry in southern California. *Trupanea nigricornis* is generalist, infesting at least 8 tribes in the Asteraceae while *T. bisetosa* is mainly a specialist on wild sunflowers. Although the two species are very similar in morphology and biology, genetic differentiation between them was possible using isozyme electrophoresis. Among 14 resolved loci, 5 were polymorphic. Significant allele frequency differences were found between the two species for PGI, PGM, ME, and EST-1. Moreover, a fixed allele difference for the locus ACPH was detected, indicating absence of gene flow between *T. nigricornis* and *T. bisetosa* in the sampled sympatric populations. The absence of hybrids could be explained by the results of cross-mating studies, which indicated that the two species did mate in the laboratory, but produced few, if any, viable offspring. Hybrid inviability acted as a post-mating barrier reducing gene flow between sympatric populations of *T. nigricornis* and *T. bisetosa*.

Key Words: Isoenzyme electrophoresis, cross-mating studies, sympatry, Tephritidae, *Trupanea*, post-mating isolating barriers

Trupanea nigricornis (Coquillett) and *T. bisetosa* (Coquillett) are flower head infesting tephritids that occur in sympatry in southern California. They are sibling and cryptic species as both their adult and immature stages show great morphological similarities and are difficult to separate (Foote et al. 1993, Knio et al. 1996a). The males can only be distinguished by the color of the third antennal segment, which is brown in *T. nigricornis* and yellow in *T. bisetosa.* Females are more difficult to separate based on anatomy. Most (ca. 75%) can be recognized by the shape of the Y-shaped apical marking on the wing. This marking is usually thin and distinct in *T. nigricornis* females and broad and short in *T. bisetosa* females (Cavender and Goeden 1983, Foote et al. 1993).

Resource utilization studies showed that the larvae of both species exploited the flower heads of their hosts in a similar manner and they fed on a similar number of achenes, taking into account the size of their host (Knio et al. 2001). However, ecologically, these two sympatric species showed major differences. *Trupanea ni-*

gricornis behaves as a generalist, infesting many Asteraceae hosts belonging to 8 tribes while *T. bisetosa* behaves as a narrowly oligophagous species infesting a few Asteraceae species belonging to only one tribe, the Heliantheae (Goeden 1985, 1992). Moreover, *T. bisetosa* could be considered as a specialist mainly on wild sunflowers, *Helianthus annuus* L., its most common host in southern California (Cavender and Goeden 1983).

Although the oviposition behavior differed between females of *T. nigricornis* and *T. bisetosa*, only subtle differences were detected in the courtship and mating behavior of the two species (Knio et al. 1996b). No apparent physical and temporal barriers seem to keep these two closely related species from meeting and mating in the field. The hosts of *T. nigricornis* bloom for a short period (1–2 months) during the fall or the spring; while the main host of *T. bisetosa*, *H. annuus*, is in bloom throughout the year in southern California, in the absence of frost. Nevertheless, spatial separation appears to be an important factor keeping these two species from meeting in areas of sympatry as adults of both species were observed to meet and mate on their respective host plants, and the timing of courtship displays was different. In the field, males of *T. nigricornis* were observed to exhibit their courtship behavior in the mornings while those of *T. bisetosa* exhibited courtship display in the afternoon (Knio et al. 1996b). However, in the insectary, cross-matings occurred between the two species and resulted in viable and fertile eggs (Cavender and Goeden 1983).

Because *T. nigricornis* and *T. bisetosa* are sympatric and taxonomically close species (Foote et al. 1993), but differ in their mode of herbivory and do not overlap in their host range, it is possible that they diverged from one common ancestor or that one species evolved from the other following host race formation. Biological characteristics permitting the development of new host races in sympatry include mating on the host plant, positive correlation between host and mate selection, and genetic control of host selection (Bush 1975). It is not known whether *T. nigricornis* and *T. bisetosa* meet all the biological criteria proposed by models of sympatric speciation. In addition, it is not known whether these two species hybridize in nature since there are no geographical barriers to isolate them and their host plants very often occur side by side in southern California.

This study investigates, using isoenzyme electrophoresis, whether gene flow occurs between sympatric populations of *T. nigricornis* and *T. bisetosa* in southern California and whether the two species produce viable offspring when crossed in the laboratory. It is one of a series of comparative studies intended to shed light on the attributes that enable *T. nigricornis* to be a successful generalist and *T. bisetosa* a specialist on wild sunflowers.

Materials and Methods

Insect collections.—The flies used in electrophoretic studies were reared from mature flower heads containing third instar larvae or puparia. The flower heads were placed in glass-topped, sleeve, insectary cages (34×32×35 cm) at the University of California, Riverside at 60% RH and 12/12 (LD) photoperiod from 0500–1700 h. After emergence, the flies fed on honey striped on the inner glass wall of the cage and water for 2–4 d, and then they were frozen and stored in liquid nitrogen at −180°C. Adults of *T. bisetosa* (N = 231) were reared from heads of wild sunflowers, *Helianthus annuus*; those of *T. nigricornis* were reared from heads of *Encelia farinosa* Gray (N = 252) and, in one instance, from heads of *E. virginensis* A. Nelson (N = 55). All collections were

made at locations of sympatry, i.e., where both wild sunflowers and *Encelia* spp. were growing in adjacent patches. The sites of collections were Casa Blanca and Lake Perris (Riverside Co.), CA; and, Wheeler Canyon (Inyo Co.), CA.

Electrophoresis.—Horizontal starch gel electrophoresis was conducted according to the techniques described by Berlocher (1980) and Pasteur et al. (1987). The gels contained 11.16% (48 g starch + 430 ml buffer) potato starch (Sigma Chemical Co.).

Each fly was homogenized with 40 μl 0.1% Triton 100-X grinding buffer using a motorized pestle. The homogenate was absorbed onto a 10×4 mm wick (Whatman #1 filter paper). Electrophoresis was stopped when the marker dye, bromophenol blue (0.1% in water), migrated about 9 cm toward the anode.

Following electrophoresis, the gel was sliced into three to four (2 mm) layers. Each slice was placed in a staining box, incubated at 37°C in a staining solution specific for a certain enzyme for 30–40 min. until the bands became distinct, then fixed in methanol:water:acetic acid (5:5:1) (Berlocher 1980). The alleles were designated according to their Rf value, the distance migrated by the allele divided by the distance migrated by the front or the marker dye.

Buffer systems and staining solutions.—Initial work consisted of resolving 14 enzymes using different buffer systems (Table 1). Five enzymes were found to be polymorphic in *T. nigricornis* and *T. bisetosa* populations. These were phosphoglucose mutase (PGM), phosphoglucose isomerase (PGI), acid phosphatase (ACPH), malic enzyme (ME), and esterases (EST). For this reason, the flies were routinely tested for these enzymes.

Two of these enzymes, PGM and ACPH, were run on aminopropyl morpholine citrate/ tris-citrate-EDTA buffer, pH 8.3: gel buffer containing 6.8 g/l monohydrate citric acid, 0.3 g/l EDTA (disodium salt), and aminopropyl morpholine to pH 8.5; and electrode buffer

Table 1. Enzymes analyzed in *Trupanea nigricornis* and *T. bisetosa* using different buffer systems.

Enzyme	(E.C. #)	Buffers tested[a]	# of major bands per individual[b]	*T. nigricornis*		*T. bisetosa*		
				Polymorphism	N[c]	Polymorphism	N[c]	Polymorphism
Adenylate kinase	(2.7.4.3)	**4**	3	no	35	no	32	no
Alcohol dehydrogenase	(1.1.1.1)	**1**, 2, 3	1	no	35	no	32	no
Aldolase	(4.1.2.13)	**1**, 2	1	no	24	no	20	no
Acid phosphatase	(3.1.3.2)	**1**, 2	1	no	307	no	231	fixed[d]
Esterases: locus 1	(3.1.1.1)	1, **2**, **3**	2	yes	107	yes	77	yes
Esterases: locus 2			4	no	107	no	77	no
Fructose 1,6 diphosphate	(3.1.3.11)	**1**	2	no	24	no	20	no
α-Glycerophosphate dehydrogenase	(1.1.1.8)	**1**, 3	2	no	24	no	20	no
Hexokinase	(2.7.1.1)	**2**	1	no	24	no	20	no
Isocitrate dehydrogenase	(1.1.1.42)	**1**, **3**	1	no	24	no	20	no
Malic enzyme	(1.1.1.40)	1, **2**	1	yes	121	yes	95	yes
Malate dehydrogenase	(1.1.1.37)	1, **2**	1	no	121	no	95	no
Phosphoglucose isomerase	(5.3.1.9)	1, **2**	1	yes	307	yes	231	yes
Phosphoglucose mutase	(2.7.5.1)	**1**, 2, **3**	1	yes	307	yes	231	yes

[a] 1 = Aminopropyl morpholine-citrate (pH 8.3) (Clayton and Tretiak 1972); 2 = Poulik (pH 8.7) (Selander et al. 1971); 3 = Tris-citrate-EDTA (pH 6.0); 4 = Phosphate citrate (pH 6.3) (Pasteur et al. 1987). The buffers that gave good resolution are marked in bold.

[b] For homozygous individuals.

[c] N = number of adults tested.

[d] fixed = fixed difference between populations tested.

containing 6.4 g/l monohydrate citric acid, aminopropyl morpholine to pH 5.0, tris to pH 8.3. (Clayton and Tretiak 1972). The enzymes PGI, ME and EST were run on tris-citrate (Poulik) buffer: gel buffer consisting of (0.076 M tris-0.005 M citric acid) 9.21 g tris and 1.05 g monohydrate citric acid/ 1 l distilled water pH 8.7; and electrode buffer consisting of (0.3 M borate) 18.55 g boric acid and 2.4 g sodium hydroxide/ 1 l distilled water, pH 8.2 (Selander et al. 1971). The tris-citrate-EDTA buffer was run for ca. 2.5 h at 120 volts and ca. 40 mA; whereas, the Poulik buffer was run for 3 h at 150 volts and ca. 50 mA.

The enzymes were stained following the recipes described by Pasteur et al. (1987) and Shaw and Prasad (1970).

Statistics.—A chi-square test was used to determine if any significant differences in the allele frequencies existed between or within *T. nigricornis* and *T. bisetosa* populations. It was also used to detect deviations from the Hardy-Weinberg equilibrium. Since some loci had several alleles with low frequencies, the alleles were pooled when it was necessary to ensure that no expected frequency was less than one and that no more than 20% of the expected frequencies was less than five (Zar 1984).

Cross-mating experiments.—The flies used in cross-mating experiments were reared from puparia dissected out of field-collected flower heads, *E. farinosa* for *T. nigricornis* and *H. annuus* for *T. bisetosa*.

After emergence, male and female flies of each species were separated and placed in different glass-topped, sleeved, cages in the insectary. Tightly wrapped cotton strips (ca. 2×1 cm) dipped in a solution of sucrose-yeast hydrolysate-water (7:4:10) (Tsiropoulos 1978) were affixed with adhesive tape to the inner wall surface of the cage as a food source, in addition to stripes of honey and water. The cotton strips were changed every other day. The flies were allowed to feed on the yeast hydrolysate diet for 18 d until the females reached sexual maturity (unpublished data). On day 18 after emergence, the mature flies were paired according to the assigned mate and transferred to smaller cages for the following treatments:

1. *T. nigricornis* females × *T. bisetosa* males (six pairs).
2. *T. nigricornis* males × *T. bisetosa* females (six pairs).
3. *T. nigricornis* females × *T. nigricornis* males (six pairs).
4. *T. bisetosa* males × *T. bisetosa* females (six pairs).
5. Unmated *T. nigricornis* females (six females).
6. Unmated *T. bisetosa* females (six females).

Each pair of flies or single female was placed in 850 ml clear-plastic cage fitted with a basal water reservoir and a screened lid to allow ventilation. The lid of each cage was striped with honey and contained a cotton strip dipped in the yeast hydrolysate solution. Each cage was provided with a bouquet of immature flower heads, the excised peduncles of which were emerged in the water reservoir and held in place by an absorbent cotton wick that also provided a water source for the flies. *Trupanea nigricornis* females were provided with *E. farinosa* heads while those of *T. bisetosa* were provided with *H. annuus* heads. The flower head bouquet was replaced every other day for a period of 2 months (May–June), and was collected from stems that had been covered with a fine mesh cloth to protect them from oviposition in the field.

The caged flower heads were dissected under a stereomicroscope and the number of eggs in each was recorded. The eggs were placed on a filter paper (Whatman #1) moistened with physiological saline in closed glass Petri dishes.

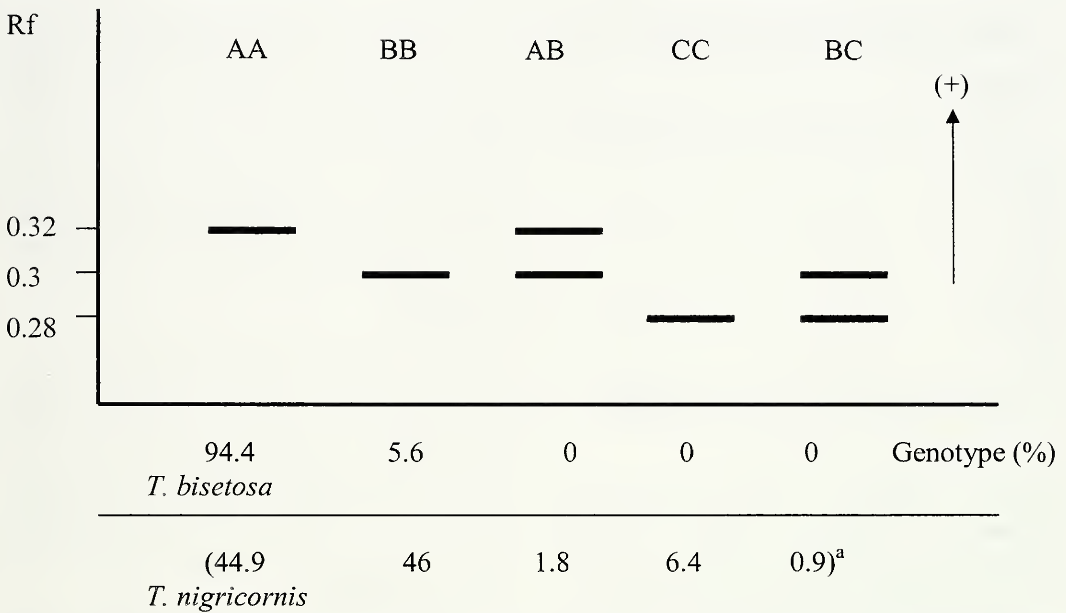

Fig. 1. Zymogram and genotypes (%) found in *Trupanea nigricornis* and *T. bisetosa* populations for malic enzyme (ME).

The Petri dishes were placed in an incubator at 27°C and checked each day for eclosion. The eggs were considered infertile if they did not hatch and remained white with no sign of embryonic development after 2 weeks, i.e., no sign of the cephalopharyngeal skeleton (Cavender and Goeden 1983). On the other hand, the eggs were considered fertile if they hatched into active first instar larvae. The number of fertile and infertile eggs was recorded and percentage eclosion for each treatment was calculated. Analysis of variance was used to compare the mean numbers of eggs laid between the different crosses and their controls.

Results

Isoenzyme electrophoresis was used to test whether gene flow occurs between sympatric populations of *T. nigricornis* and *T. bisetosa*. Table 1 summarizes the isozyme loci resolved in this study, the number of flies analyzed for each locus, the buffer systems used and the number of major bands found per locus. Among 14 resolved loci, only acid phosphatase, phosphoglucose isomerase, phosphoglucose mutase, malic enzyme, and esterases were polymorphic in *T. nigricornis* and *T. bisetosa* populations. The other tested enzymes were monomorphic indicating genetic similarity between the two species.

Among the polymorphic enzymes, ME and EST were not always clearly resolved and therefore were not included in the intraspecific variation analysis. Malic enzyme behaved as a monomer. Two alleles for ME were detected in *T. bisetosa* populations while three were detected for *T. nigricornis* populations (Fig. 1). Allele 0.32 was almost fixed for *T. bisetosa* populations with a frequency of 0.94 (Table 2). There was a significant difference in the allele frequencies of ME between the two species ($X^2 = 89.2$; df = 2; $p < 0.001$). In the esterases, two loci were detected. EST-1 was polymorphic with three common alleles in the two species (Fig. 2); significant allele fre-

Table 2. Allele frequencies of *Trupanea bisetosa* and *T. nigricornis* at the polymorphic loci, ME and EST.

Allele[a]	*T. bisetosa*	*T. nigricornis*
ME**		
0.32	0.94	0.46
0.3	0.06	0.47
0.28	0	0.07
EST-1*		
0.26	0.18	0.09
0.24	0.62	0.78
0.22	0.29	0.04

[a] Each allele is referred to by its Rf value.
* $p < 0.005$; ** $p < 0.001$.

quency differences were also detected between *T. nigricornis* and *T. bisetosa* populations for EST-1 ($X^2 = 18.1$; df = 1; $p < 0.005$) (Table 2).

The most useful enzymes for differentiating between *T. nigricornis* and *T. bisetosa* populations were ACHP, PGI, and PGM. Acid phosphatase was fixed for allele 0.8 in *T. nigricornis* and fixed for allele 0.9 in *T. bisetosa* in all samples analyzed (Fig. 3). The absence of hybrids indicated that mating did not occur between the two species in the populations that were sampled. This locus could be considered diagnostic as it allows discrimination between the two sympatric species.

Two other loci, PGM and PGI, showed genetic differentiation between *T. nigricornis* and *T. bisetosa*. Allele frequencies for PGM and PGI were significantly different between the two species ($p < 0.001$) (Table 3). However, there was no significant allele frequency difference within samples of the same species (Table 3).

The polymorphic locus PGM, which behaved as a monomer, showed greater genetic variability in *T. nigricornis* popu-

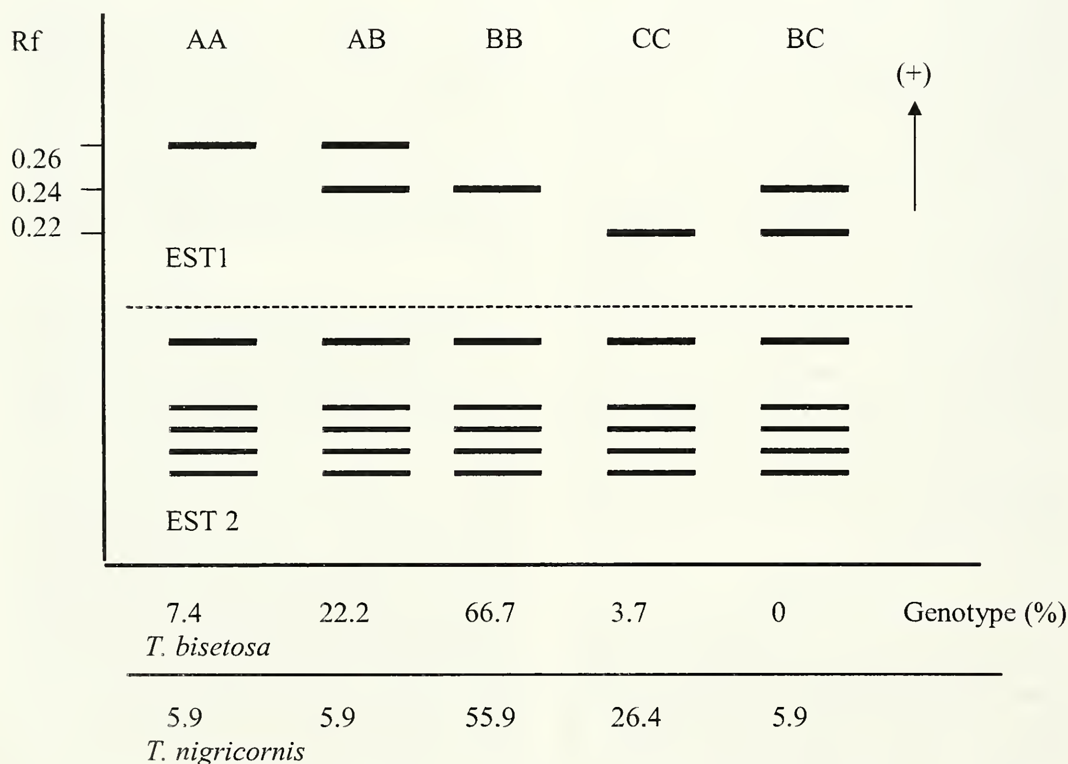

Fig. 2. Zymogram and genotypes (%) found in *Trupanea nigricornis* and *T. bisetosa* populations for esterases (EST), loci 1 and 2.

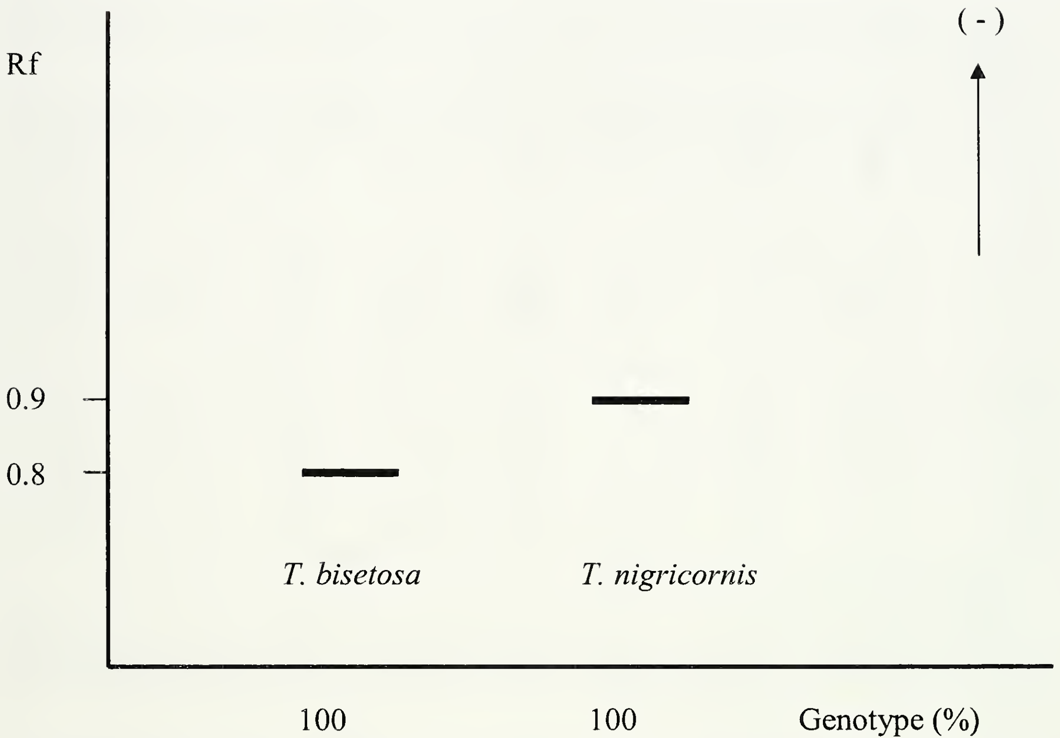

Fig. 3. Zymogram of acid phosphatase (ACPH) in *Trupanea nigricornis* and *T. bisetosa* populations, showing a fixed allele difference between the two species.

lations than in *T. bisetosa* populations. Four alleles for that locus were detected in *T. bisetosa* populations; six alleles were detected in *T. nigricornis* populations. The mean number of alleles was 2.67 in *T. bisetosa* samples and 5.00 in *T. nigricornis* samples. A summary of all PGM phenotypes and their genetic interpretation is presented in Fig. 4. The two species shared the alleles 0.09, 0.12, 0.16, and 0.19. Allele 0.12 was the most common allele in both species, with a total frequency of 0.964 in *T. bisetosa* and 0.609 in *T. nigricornis* (Table 3). Two rare alleles (0.06 and 0.10) were only detected in *T. nigricornis* samples from flies reared from *E. farinosa* flowerheads, but not *E. fructescens* heads. Moreover, PGM was almost fixed in three *T. bisetosa* samples with the frequency of the most common allele (0.12) greater than 0.95 (Table 3).

This greater genetic variation for PGM seemed to be preserved in *T. nigricornis* populations since chi-square tests on expected and observed genotypic frequencies indicated that the PGM locus did not depart from the Hardy-Weinberg equilibrium (Table 4). The Hardy-Weinberg test was not applied to *T. bisetosa* because the locus was almost fixed for the most common allele, leaving the other alleles with genotypic classes having low frequencies (<1), even after pooling.

The polymorphic locus, PGI, behaved as a dimer. It also showed genetic variability between the species. Two alleles were detected in *T. bisetosa*; five were detected in *T. nigricornis*. A mean of 1.33 alleles per locus was found in *T. bisetosa* samples while a mean of 3.33 alleles per locus was found in *T. nigricornis* samples. The phenotypes observed for PGI in the two species are summa-

Table 3. Allele frequencies of *Trupanea bisetosa* and *T. nigricornis* at two polymorphic loci, PGI and PGM.

Allele[a]	Allele frequency in *T. bisetosa*[c]				Allele frequency in *T. nigricornis*[c]			
	Site 1	Site 2	Site 3	Total	Site 1	Site 2	Site 3	Total
N[b]	155	58	18	231	130	142	35	307
PGI								
0.4	0	0	0	0	0	0.004	0	0.002
0.33	0	0	0	0	0	0.007	0	0.003
0.25	0.006	0	0	0.004	0.042	0.049	0.014	0.042
0.17	0.994	1	1	0.996	0.954	0.930	0.986	0.946
0.09	0	0	0	0	0.004	0.011	0	0.007
PGM								
0.19	0.010	0.009	0	0.009	0.027	0.007	0.043	0.020
0.16	0.019	0.034	0	0.022	0.173	0.162	0.129	0.163
0.12	0.952	0.922	0.972	0.946	0.6	0.627	0.571	0.609
0.10	0	0	0	0	0	0.011	0	0.005
0.09	0.019	0.034	0.028	0.024	0.192	0.155	0.257	0.182
0.06	0	0	0	0	0.008	0.039	0	0.021

[a] Each allele is referred to by its Rf value.
[b] Number of adults tested.
[c] Within *T. bisetosa*: $X^2 = 0.99$ (df = 2; $p > 0.05$) for PGI and $X^2 = 2.7$ (df = 4; $p > 0.05$) for PGM. Within *T. nigricornis*: $X^2 = 4$ (df = 2; $p > 0.05$) for PGI and $X^2 = 5.9$ (df = 6; $p > 0.05$) for PGM. Between species: $X^2 = 20.4$ (df = 1; $p < 0.001$) for PGI and $X^2 = 161.2$ (df = 3; $p < 0.001$) for PGM. Alleles were pooled to maintain expected allele frequency >1.

rized in Fig. 5; the frequencies of the PGI alleles are shown in Table 3. The two species shared allele 0.17, the most common allele, and allele 0.25, which occurred at low frequencies (total f = 0.0042) in *T. nigricornis* samples and in lower frequencies (total f = 0.004) in two out of six *T. bisetosa* samples. The locus PGI appears fixed in *T. bisetosa* populations as the frequency of the most common allele ranged from 0.99–1.00 (Table 3). The frequency of the most common allele was also high (0.99) in one *T. nigricornis* sample from flies collected from *E. fructescens* flower heads. Testing for the Hardy-Weinberg equilibrium was not possible for PGI in both species because this locus was almost fixed for the most common allele.

Cross-mating tests between *T. nigricornis* and *T. bisetosa* revealed that the two species did mate in the laboratory, but produced few viable offspring. When *T. nigricornis* females were crossed with *T. bisetosa* males, they produced an average of 66 eggs per female in a period of 2 months; however, only ca. 1% of the eggs were fertile and hatched into first instar larvae (Table 5). When *T. bisetosa* females were crossed with *T. nigricornis* males, they oviposited an average of 16 eggs per female in 2 months, but none of these eggs were fertile, i.e., percent eclosion was zero (Table 5). Eclosion was 100% in the control crosses, *T. nigricornis* × *T. nigricornis* and *T. bisetosa* × *T. bisetosa* (Table 5). Eclosion was 0% when *T. nigricornis* females and *T. bisetosa* females were reared without males. The unmated females produced very few eggs, and none were fertile (Table 5).

Analysis of variance showed significant differences in the mean number of eggs laid by *T. nigricornis* and *T. bisetosa* females in the cross-mating tests ($F = 34.65$; df = 35; $p < 0.05$). *Trupanea nigricornis* females crossed with either *T. nigricornis* males or *T. bisetosa* males produced significantly (95% level) more

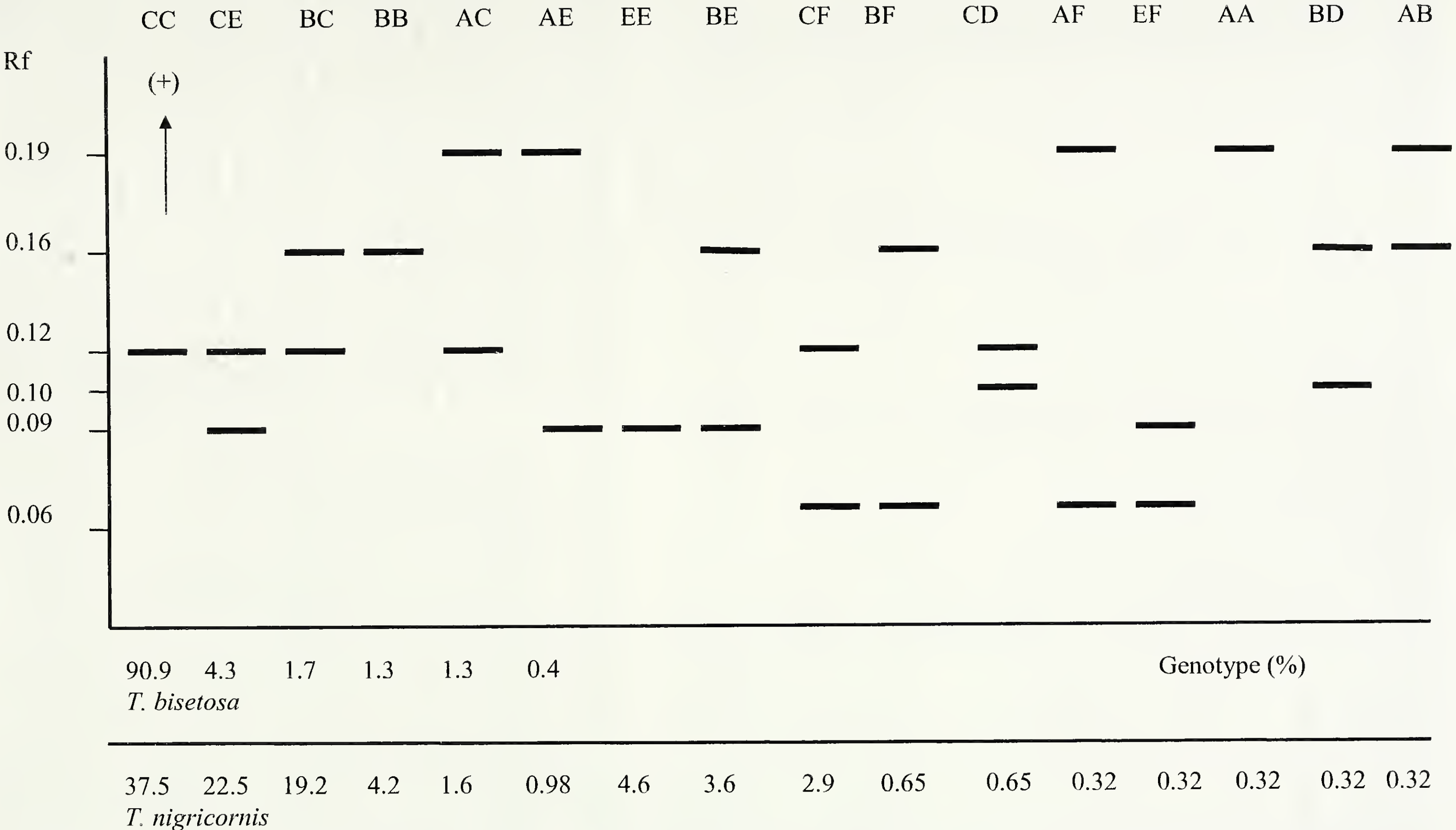

Fig. 4. Zymogram and genotypes (%) found in *Trupanea nigricornis* and *T. bisetosa* populations for the enzyme phosphoglucose mutase (PGM).

Table 4. Test for Hardy-Weinberg equilibrium at the PGM locus in *T. nigricornis* populations. Alleles were pooled to maintain expected genotypic frequency > 1.

Population	Genotypes[a]	Genotypic frequencies		Chi-square
		Observed	Expected	
Site 1:				
	CC	46	46.8	0.01
	CE	33	29.95	0.31
	BC	31	32.45	0.07
	BB	8	5.62	1.00
	BE	7	10.38	1.10
	EE	5	4.79	0.01
		130	129.99	2.50[a]
Site 2:				
	CC	59	55.82	0.18
	CE	33	37.75	0.60
	BC	27	28.85	0.12
	EE	10	6.38	2.05
	BE	7	9.75	0.78
	BB	6	3.73	1.39
		142	142.28	5.12[a]
Site 3:				
	CC	10	11.41	0.17
	CE	20	17.15	0.48
	EE	5	6.44	0.32
		35	35	0.97[a]

[a] $p < 0.05$.

eggs than *T. bisetosa* females crossed with *T. nigricornis* males or males of their own species (Table 5). There was no significant difference in the mean number of eggs laid between *T. nigricornis* females crossed with *T. bisetosa* males and the controls. However, *T. bisetosa* females crossed with *T. nigricornis* males produced significantly less eggs than the controls (Table 5). The mean number of eggs produced by the unmated females did not differ between the two species. However, it was significantly lower than the means of mated females of both species in the crosses and the control tests (Table 5).

Discussion

The sympatric and cryptic species, *T. nigricornis* and *T. bisetosa*, showed much genetic similarity. Ten of the 14 resolved loci were monomorphic in the two species. Nevertheless, genetic differentiation between the two species was possible as there was a fixed allele difference for the locus ACPH, and significant allele frequency differences for the loci, PGI, PGM, ME, and EST-1. The fixed allele difference for the diagnostic locus ACPH indicates that the two sympatric species do not hybridize in nature as no hybrids were detected in the populations that were sampled. Hence, there should be some reproductive isolation mechanisms that restrict gene flow between the two species.

Two types of reproductive barriers seem to be involved in the prevention of gene flow between *T. nigricornis* and *T. bisetosa*. These are pre-mating and post-mating isolating barriers. The first type, pre-mating barriers, can operate through ethological isolation and differ-

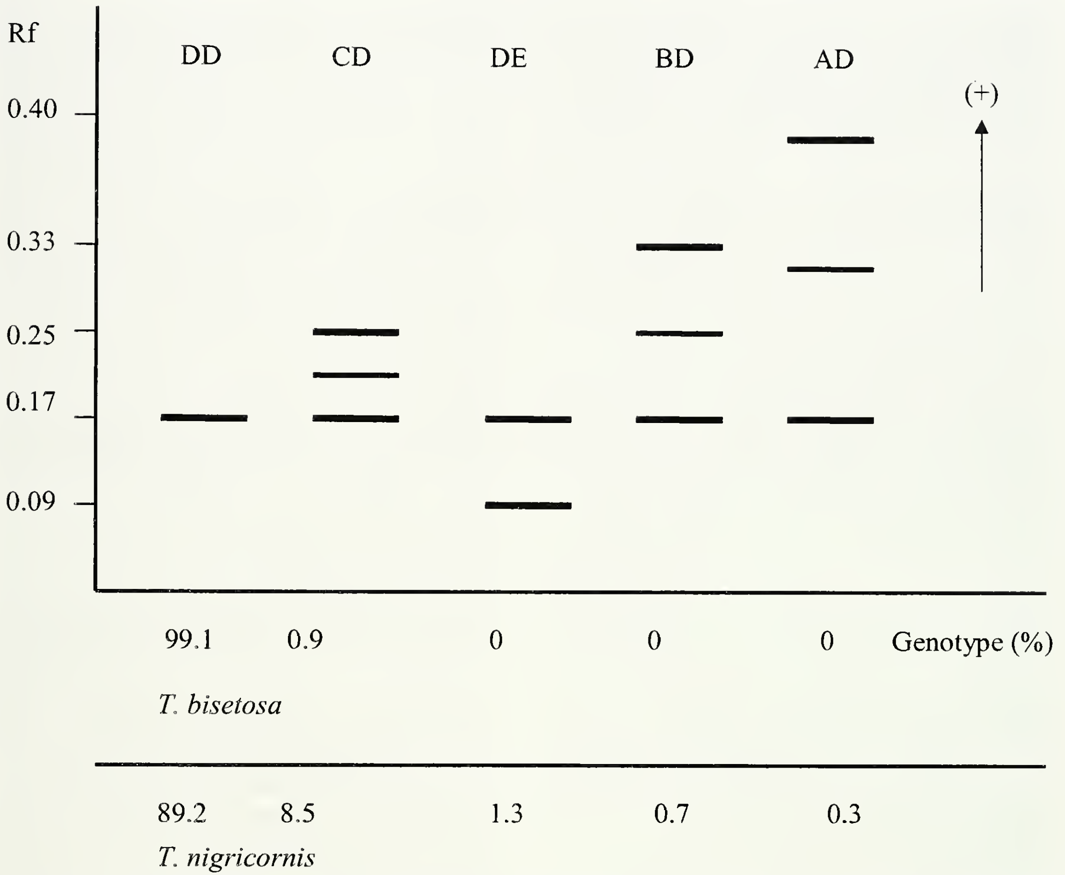

Fig. 5. Zymogram and genotypes (%) found in *Trupanea nigricornis* and *T. bisetosa* populations for the enzyme phosphoglucose isomerase (PGI).

ential host recognition. Ethological mechanisms include a few differences in the courtship behavior of *T. nigricornis* and *T. bisetosa*, especially differences in the wing movements of the males (Knio et al. 1996b). However, because males of both species have very similar wing patterns and because most successful matings are force matings while the females are probing the flower heads

Table 5. Percent eclosion in *T. nigricornis* (T. n.) and *T. bisetosa* (T. b.) crosses and controls.

Crosses (Female × Male)	N[a]	# of eggs laid	Fertile Eggs	Eclosion (%)
		Mean[b] ± (SE)	Mean	
T. n. × T. b.	6	99 (12.2) a	1.11	1.69
T. b. × T. n.	6	29.8 (8.3) b	0	0
T. n. × T. n.	6	119.8 (11) a	119.8	100
T. b. × T. b.	6	62 (8.4) c	62	100
T. n. × -	6	2.8 (1.1) d	0	0
T. b. × -	6	2.2 (0.9) d	0	0

[a] N = number of adult pairs.
[b] Means followed by the same letter are not significantly different, $p < 0.05$, as determined by the least significant difference.

(Knio et al. 1996b), differential host recognition is probably more significant than ethological isolation in restricting gene flow. Similarly, in the sibling species *Rhagoletis mendax* Curran and *R. pomonella* (Walsh) (Diptera: Tephritidae) that court and mate on or near their respective host plants (Prokopy et al. 1971, 1972), differences in oviposition preference and in host recognition were the most important pre-mating barriers reducing hybridization between these two sympatric species (Feder et al. 1989). Because *R. mendax* and *R. pomonella* readily mate in the laboratory and field experiments producing viable offspring, differential host usage and host fidelity were found to be more significant in reducing contact between the two species than post-mating and ethological isolation barriers (Feder and Bush 1989). Moreover, in the apple and hawthorn races of *R. pomonella*, differential host recognition by the adult flies together with differences in the timing of adult emergence were responsible for reducing gene flow and causing rapid genetic differentiation between these sympatric races (Feder et al. 1988, Prokopy et al. 1988). In fact, mark-release and recapture experiments demonstrated that host fidelity was an efficient barrier to gene flow between the apple and hawthorn races reducing hybridization to about 6% per generation (Feder et al. 1994). Factors that contributed to this host fidelity were genetically based differences in host preference, adult eclosion under the specific host, and more importantly, allochronic isolation of the adults due to differences in the phenology of their respective plant hosts (Feder et al. 1988, 1994). Contrary to the univoltine *R. pomonella* host races, it is unlikely that seasonal asynchrony is an important factor in reducing gene flow between the multivoltine tephritids, *T. nigricornis* and *T. bisetosa* because there is a large overlap in the flowering period of their different hosts. Although wild sunflowers bloom throughout the year in southern California, the highest densities of the specialist, *T. bisetosa*, are in the spring and in the fall, coinciding with the blooming seasons of the hosts of the generalist, *T. nigricornis*.

Post-mating isolating mechanisms are also involved in restricting gene flow between *T. nigricornis* and *T. bisetosa*. The crosses between the two species yielded eggs with zero to very low eclosion. Since females in these cross-mating tests oviposited significantly more eggs than unmated females of either species, this indicates that the two species cross-mated but produced infertile eggs. Therefore, post-mating barriers are reducing gene flow through hybrid inviability. They also explain why no hybrids for ACPH were detected in the populations analyzed. In case of host identification mistake and mating with a nonconspecific male, post-mating barriers are thus effective in isolating the two species because the hybrids produced are not likely to survive. Post-mating isolating barriers were also found to play a role in limiting hybridization between *R. mendax* and *R. pomonella* through a reduction in the viability of the hybrids (Feder et al. 1989, Feder and Bush 1989). Recently, it was demonstrated that F_1 hybrids resulting from crosses between the apple and hawthorn races of *R. pomonella* in addition to a sister species infesting dogwood showed great fitness disadvantage in finding potential fruit hosts as they failed to respond to fruit volatiles at concentrations causing maximal orientation of their respective parents (Linn et al. 2004).

In conclusion, the electrophoretic data supports the differences in behavior and biology observed in *T. nigricornis* and *T. bisetosa*. The two species are very close morphologically and genetically, but they do not hybridize in nature. The two species meet most of the criteria required

for sympatric speciation. Mechanisms that reduce gene flow between these two sympatric species include meeting and mating on different host plants, differences in host recognition, and post-mating sterility through null hybrid viability. The key to host selection in *T. nigricornis* and *T. bisetosa* appears to be in the behavior of adult females rather than in survival of the immatures in suitable hosts. The oviposition behavior of the females coupled with their adaptation to the biophysical features of their host plants are the most important factors determining differences in host uses and host specificity of these species (Knio et al. 1996b, 2001). The trade-off for adopting a narrow host range and specializing on wild sunflowers in *T. bisetosa* seem to be gaining a greater enemy-free space (Knio et al. 2007).

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